United States Department of Agriculture
Animal and Plant Health Inspection Service
Plant Protection and Quarantine

Water Sampling for *Phytophthora ramorum* in Infested or Potentially Infested Nurseries

Adapted from protocols in use by the Oregon Department of Agriculture

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Phytophthora ramorum is an oomycete, belonging to the group that includes *Pythium* species. Collectively these organisms are called "water molds" and are taxonomically related closer to algae than to fungi. For this reason, water collected from potentially infested nursery blocks must be tested for the presence of *P. ramorum*.

There are two potential methods provided here to detect *Phytophthora* species in water. The first uses rhododendron leaf baits in mesh bags followed by moist chamber incubation of the leaf baits. Any suspect lesions that develop on the rhododendron leaves would be plated on PARP. Any *Phytophthora* species growing on the PARP would need to be transferred to Corn meal agar or V8 agar for identification to species.

The second method uses water filtration. Water is removed from the pond, filtered with sterile filters and the filters placed on PARP. Once the filter is removed from PARP, any resultant *Phytophthora* colonies are transferred to Corn Meal Agar or V8 agar and identified to species.

In situ Water Sampling with Rhododendron Leaf Baits

Prepare the rhododendron leaves as bait by cutting the leaves in a herringbone pattern into (but not through) the mid-vein or by trimming off the petiole end of each leaf. Place 3-4 cut leaves into a mesh bag. Label the bag with a plastic tag listing the date, water source (location), and nursery (i.e., nursery license number). Place the mesh bag into the water source for a minimum of 48-h to 1-wk (preferable). Do not leave the bait in the water source for longer than 1-wk as the bait will begin to decompose. Place the bags such that the leaves will remain submerged the entire time (i.e., even if water levels fluctuate within the water source). If possible, place the bait near the influent coming from the area closest to or containing the infested plants.

Remove the bait from the water source and transfer to a sealable bag for transport to the laboratory. Label the bag with the information on the plastic tag, including the date collected. Log the leaf samples into the appropriate database. Assign a unique sample number to the bait(s) from each nursery.

Water Sampling for Filtration

Water samples should be collected in a sterile wide-mouth bottle and kept at 5 – 10 C. Water samples should be taken from the surface to increase the likelihood of obtaining zoospores of Phytophthora.

Sample size should be approximately 1000 ml. Sample should be processed within 48 hours of collection. Number of samples is determined by the size of the nursery pond to be sampled (Table 1)

Table 1. Number of composite samples collected based on pond size.

Size of pond (acres)	No. of water samples collected (liters)
0.00 - 0.25	1
0.26 - 0.5	2
0.50 - 1.0	4
1.01 - 2.50	8
>2.51	12

Processing of Samples in the Laboratory

Have prepared plates of PARP (see the USDA approved *Guidelines for Isolation by Culture and Morphological Identification of Phytophthora ramorum [http://www.aphis.usda.gov/ppq/ispm/sod/cultureprotocol.html])* and autoclave or obtain sterilized 5 µm pore size, 47 mm diameter nitrocellulose or polycarbonate membrane filters an 47 mm filter funnel apparatus. Sterilize filter forceps in alcohol and flame before each use.

Label all tubes and plates with appropriate sample identification.

Set up filter funnel in receiving apparatus, e.g., side-arm flask or manifold connected to a large volume reservoir. Make sure there is a secondary trap between the receiving apparatus and the vacuum source (water aspirator or mechanical pump). When using the manifold, turn on vacuum with all manifold valves closed. When using a side-arm flask, leave vacuum off until water sample has been placed in the filter funnel. Place membrane filter on funnel base using flamed forceps and clamp on filter funnel. Seat funnel and clamp securely to avoid leaks.

Gently agitate water sample by swirling and inverting sample bottle and pour 100 ml into the funnel. This is usually the largest volume of surface water that will filter in a reasonable amount of time. If the water sample is excessively turbid, it may be necessary to use 10 ml volumes.

Open manifold valve or turn on vacuum to filter sample. As soon as the entire sample has passed through the membrane filter, immediately close the manifold valve or turn off the

vacuum to avoid overdrying the specimen. Unclamp the filter funnel and remove membrane with flamed forceps. Place membrane face-down on the surface of the agar plate (PARP).

Amount of sample to filter, or amount of serial dilution is determined by expected number of propagules, or by desired detection limit. This should be determined ahead of time to ensure adequate preparation, and efficient use of resources.

Filter Processing

Filters may be plated out on PARP or ELISA may be used (see the USDA approved *Guidelines for Isolation by Culture and Morphological Identification of Phytophthora ramorum [http://www.aphis.usda.gov/ppq/ispm/sod/cultureprotocol.html]* and USDA approved *ELISA Protocol [http://www.aphis.usda.gov/ppq/ispm/sod/ELISAprotocol.html])*. If ELISA is used to prescreen, any positive ELISA reaction must be plated out on PARP or must follow the Nested PCR protocols (*see Confirmed Nursery Protocol [http://www.aphis.usda.gov/ppq/ispm/sod/nursery.html])*.

If plating the filters, inoculated plates should be incubated at 22 C in the dark. (Incubation at 18 C to 22 C is acceptable). Use a reliable incubator if room temperature is unpredictable.

After 24 hours remove membranes from agar surface with flamed forceps and discard. Examine plates for colony growth at this time and at subsequent 24 hour intervals. Use microscope to check for septation. Colonies with coenocytic hyphae should be subcultured to confirm identity as *Phytophthora* or *Pythium*. Later forming coenocytic colonies (usually after 4 – 5 days) are often Mucorales, and must also be ruled out.

Leaf Bait Processing

If multiple baits are used, treat each as a subsample for that nursery. Set the bait on a moistened paper towel inside a sealable container. Incubate at room temperature to let any potential fungal cultures develop.

Examine each rhododendron leaf daily for developing symptoms. The *P. ramorum* symptom that forms is usually a brown leaf spot with a diffuse margin. The lesion may or may not be more extensive along the mid-vein of the leaf. From the edge of each developing lesion, remove tiny pieces of rhododendron leaf with a sterile scalpel blade and transfer to PARP medium. Place six to eight pieces from each leaf onto a PARP plate.

Identify any *Phytophthora* species (see the USDA approved *Guidelines for Isolation by Culture and Morphological Identification of Phytophthora ramorum [http://www.aphis.usda.gov/ppq/ispm/sod/cultureprotocol.html])*. If *P. ramorum* is identified please follow the confirmed nursery protocols.

This protocol, in one form or another, has been used for decades to isolate Phytophthora species from soil and/or water samples, however, this protocol has not been completely validated by PPQ and may be adjusted periodically as the validation process is undertaken.